(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 August 2001 (23.08.2001)

PCT

(10) International Publication Number
WO 01/60998 A2

(51) International Patent Classification[7]: C12N 15/11, A61P 35/00, A61K 48/00, 9/127

(21) International Application Number: PCT/US01/40159

(22) International Filing Date: 20 February 2001 (20.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/506,979 18 February 2000 (18.02.2000) US

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(81) Designated States *(national)*: CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *without international search report and to be republished upon receipt of that report*

WO 01/60998 A2

(54) Title: SMALL OLIGONUCLEOTIDES WITH ANTI-TUMOR ACTIVITY

(57) Abstract: The present invention provides short antisense oligonucleotide compositions and methods for their use in the treatment of Bcl-2-associated diseases like cancer, such as follicular lymphoma (FL). The antisense oligonucleotides contain sequences that hybridize to Bcl-2 nucleic acids, the gene products of which are known to interact with the tumorigenic protein Bcl-2. The use of novel short antisense oligonucleotides, from 7 bases to 9 bases in length, is described in this invention. The invention also describes certain specific sequences which are longer than 9 bases and are 11 or 15 bases long. Used alone, or in conjunction with other antisense oligonucleotides, these antisense oligonucleotide compositions inhibit the proliferation of cancer cells.

DESCRIPTION

SMALL OLIGONUCLEOTIDES WITH ANTI-TUMOR ACTIVITY

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the fields of cancer therapy. More particularly, the invention concerns the use of small antisense oligodeoxynucleotides for antitumor therapy.

2. Description of Related Art

The *bcl-2* gene has been associated with a wide variety of diseases such as hematologic malignancies and includes both leukemias and lymphomas and more specifically includes follicular and nonfollicular lymphomas, chronic lymphocytic leukemia, and plasma cell dyscrasias (Campos *et al.*, 1994); solid tumors like those associated with breast, prostate and colon cancer; and immune disorders. One particular Bcl-2-related disease is Follicular non-Hodgkin Lymphoma (FL). Follicular lymphoma is the most common lymphoid malignancy in Europe and the United States. Typically it is an indolent, low grade disease consisting of an accumulation of small, resting B cells. Although the initial response to chemotherapy is good, relapses are inevitable and the disease transforms to a more aggressive histological type and develops drug resistance (Aisenberg, 1995; Johnson *et al.,* 1995).

In over 90% of follicular lymphoma patients, a t(14;18) translocation is found, which results in the juxtaposition of the *bcl-2* gene from chromosome 18q21 with the immunoglobulin heavy chain gene locus on chromosome 14q323 (Tsujimoto *et al.,* 1985; Graninger *et al.,* 1987). As a consequence. the *bcl-2* gene comes under the influence of an immunoglobulin heavy chain enhancer, resulting in the overexpression of the Bcl-2 protein (Bakhshi *et al.,* 1985; Tsujimoto *et al.,* 1987). The tumorigenic potential of Bcl-2 is related to its capacity of interfering with physiological cell-death responses, thereby enhancing the longevity of the cell (Nuñez *et al.,* 1990). The Bcl-2 protein blocks apoptotic stimuli such as growth factor deprivation, radiation, heat-shock, virus, and most DNA damaging agents for example, most chemotherapeutic agents (Reed, 1995; Hockenbery *et al.,* 1990). In *bcl-2*-Ig-transgenic mice, a polyclonal follicular lymphoproliferation consisting of an expansion of mature B lymphocytes is initially observed

(McDonnell *et al.,* 1989). Subsequently, monoclonal high grade large immunoblastic type lymphomas develop and about 50% of them present rearrangement of C-MYC. This suggests that a second genetic alteration is necessary for the development and progression of malignant lymphoma (McDonnell and Korsmeyer, 1991).

An expanding family of Bcl-2-related proteins have been identified and include Bax, Bcl-X_L, Bcl-X_S, Bad, Bak, Mcl-1, A-1, and several open reading frames of DNA viruses (Oltvai *et al.,* 1993; Boise *et al.,* 1993; Yang *et al.,* 1995; Chittenden *et al.,* 1995; Kiefer *et al.,* 1995; Kozopas *et al.,* 1993; Lin *et al.,* 1993; Pearson *et al.,* 1987; Neilan *et al.,* 1993). Membership in the Bcl-2 family of proteins is principally defined by homology within the BH1 and BH2 domains, which help regulate dimerization between the members (Sato *et al.,* 1994). Bax, which shares 21% amino-acid identity with Bcl-2, can bind to Bcl-2 protein and neutralize its ability to block cell death. Thus, the ratio of Bcl-2 to Bax is thought to determine the cell's susceptibility to death following an apoptotic stimulus (Oltvai *et al.,* 1993; Yin *et al.,* 1994). U.S. Patent No. 5,837,838 to Reed *et al.*, 1998c, provides methods for identifying agents that can modulate the binding of a Bax-inhibitor protein to a member of the Bcl-2 family of proteins.

Phosphodiester antisense oligodeoxynucleotides complementary to specific sequences of the translation-initiation site of Bcl-2 mRNA are able to inhibit the production of the Bcl-2 protein and thereby inhibit the growth of t(14;18) translocation bearing cells (Kitada *et al.,* 1993). However, therapeutic use of phosphodiester oligonucleotides is hampered by their low cellular uptake and their rapid degradation by nucleases and other serum or cellular components. Phosphorothioate oligonucleotides, which are resistant to nuclease degradation, were found to inhibit follicular lymphoma cell growth at concentrations 10 times lower than phosphodiester oligonucleotides (Reed *et al.,* 1990a; Reed *et al.,* 1990b; Cotter *et al.,* 1994). However, this approach suffers from low cellular uptake of the oligonucleotides. For example, Reed *et al.,* (1990a) and Reed *et al.,* (1998a), had to use concentrations of greater than 25μM of phosphorothioates to achieve 50% growth inhibitions of human leukemic cell-lines Su-Dhl-4, RS11846, 679 and JURKAT and in human PBL (peripheral blood lymphocytes).

Incorporation of oligonucleotides into liposomes has increased their uptake into leukemic cells (Akhtar *et al.,* 1991; Tari *et al.,* 1994). The use of cationic lipids by Reed *et al.,* to deliver phosphorothioate antisense oligonucleotides allowed them to reduce the concentration of oligonucleotides to 0.075 to 0.3 μM and still induce growth inhibition in Su-Dhl-4 cells.

In a related invention, disclosed in U.S. Patent Application Serial No. 09/112,869, filed July 9, 1998, the present inventors describe various liposomal compositions of antisense

oligonucleotides and methods of making these compositions. The application also describes the use of these liposomal compositions to deliver antisense oligonucleotides to tumor cells and methods for inhibiting the growth of tumor cells.

U.S. Patent No. 5,734,033 (Reed *et. al.,* 1998a), reports the use of antisense oligonucleotide sequences derived from regions of the translation-initiation site of the *bcl-2* gene which are 10 bases or greater in length for the inhibition of growth of leukemic cells and human PBL cells. However, there are no cxamples demonstrating the synthesis and use of antisense oligonucleotides shorter than 15-mers. Also, both the phosphorothioate and the phosphodiester antisense oligonucleotides were required at concentrations greater than 25μM for the inhibition of about 50% of cell growth in the human lcukcmic cell-lines RS11846, 679 and JURKAT and in human PBL (peripheral blood lymphocytes).

Related U.S. Patent No. 5,831,066 to Reed (1998b), proposes that antisense oligomers of from 2 to 200 nucleotides in length will bind to a human *bcl-2* mRNA at the translation initiation site and reduce *bcl-2* expression in tumor cells. However, again, there is no disclosure reciting the synthesis and successful use of antisense oligonucleotides shorter than 15 mers.

There is, therefore, a great need for better compositions for the treatment of Bcl-2 associated diseases such as hematologic malignancies, both leukemias and lymphomas, including follicular and nonfollicular lymphomas, chronic lymphocytic leukemia, and plasma cell dyscrasias; solid tumors like those associated with breast, prostate and colon cancer; and immune disorders.

SUMMARY OF THE INVENTION

The present invention overcomes these and other deficiencies in the art and demonstrates the use of very short *bcl-2* antisense oligonucleotides in lipid formulations, ranging from 7 bases to 9 bases, that induce growth inhibition in human leukemic cells. This is an unexpected result in light of U.S. Patent No. 5,734,033, to Reed *et al.*, 1998a, which reports a lower limit of 10 bases for the antisense oligonucleotides. It is well known to one of skill in thc art, that while all or part of a gene sequence may be employed in the context of antisense construction, statistically, any sequence of at least 17 bases long should occur only once in the human genome and, therefore, be essential to specify a unique target sequence. In light of this knowledge, it is surprising that in the present invention, short oligonucleotides, defined herein as oligonucleotides of 9 or lcss bases in length, such as oligonucleotides 9 bases, 8 bases and/or 7 bases in length,

have been used with success as specific antisense molecules towards *bcl-2*. In contrast to conventional wisdom, which dictates that both binding affinity and sequence specificity of an oligonucleotide for its complementary target, increase with increasing length, the inventors have demonstrated the successful use of short oligonucleotides.

Additionally, in the present invention, the concentrations of the short antisense oligonucleotides in lipid formulations, for example, the short oligonucleotides represented by SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5, used to achieve 50% growth inhibition in leukemic cells are low and range from about 3 to 12μM. This result is also true for the oligonucleotides of this invention which are longer than 9 bases. Specifically, two 11 base long oligonucleotides represented by SEQ ID NO: 6 and SEQ ID NO: 7; and two 15 base long oligonucleotides represented by SEQ ID NO: 8, and SEQ ID NO: 9 are very efficient in controlling the growth of a human leukemia cell line and are required at a concentration of only 3 to 4μM to achieve 50% growth inhibition.

The findings of Reed *et. al.*, 1998a, which reports the use of *bcl-2* antisense phosphorothioate oligonucleotides of 10 bases or greater, which are not liposomal formulations, require concentrations greater than 25μM for the inhibition of 50% of cell growth in some human leukemic cell-lines and in human PBL (peripheral blood lymphocytes). Furthermore, in Reed *et al.*, 1998b, concentrations of greater than 60μM are required for about 50% growth inhibition of leukemic cells.

Thus, in one embodiment, the present invention provides a composition comprising a short antisense oligonucleotide, of from seven bases to nine bases in length, that is complementary to a Bcl-2 oligonucleotide, and a lipid component. In a further embodiment, the oligonucleotide includes a region that is complementary to a portion of, or overlaps with a portion of, the translation initiation site of Bcl-2 mRNA. In certain specific embodiments, the oligonucleotide will include the sequence SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5.

In another aspect of the invention, the oligonucleotide may be greater than nine bases in length. In a specific embodiment of this aspect, the oligonucleotide is 11 or 15 bases long and has the sequence corresponding to SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9.

In one aspect, the lipid component of the composition comprises liposomes. In another aspect the short antisense oligonucleotide is encapsulated in liposomes, interspersed within the

lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, complexed with a lipid, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid.

The term "lipids" as used in this specification and the claims denotes any form of both naturally occurring and synthetic lipids or liposomes. They are fatty substances and are well-known to those of skill in the art. The lipids of the present invention are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates which are not uniform in either size or shape.

In a preferred embodiment, the lipid material is comprised of a neutrally charged lipid. A neutrally charged lipid can comprise a lipid without a charge, a substantially uncharged lipid or a lipid mixture with equal number of positive and negative charges.

In one aspect, the lipid component of the composition comprises a neutral lipid. In another aspect, the lipid material consists essentially of neutral lipids which is further defined as a lipid composition containing at least 70% of lipids without a charge. In other preferred aspect, the lipid material may contain at least 80% to 90% of lipids without a charge. In yet other preferred aspects, the lipid material may comprise about 90%, 95%, 96%, 97%, 98%, 99% or 100% lipids without a charge.

The preferred lipid in the present invention is comprised of dioleoylphosphatidylcholine. However, other lipids such as phosphatidylcholines, phosphatidylglycerols, and phosphatidylethanolamines may also be employed.

In other aspects the lipid component comprises a substantially uncharged lipid. A substantially uncharged lipid is described herein as a lipid composition that is substantially free of anionic and cationic phospholipids and cholesterol. In yet other aspects the lipid component comprises a mixture of lipids to provide a substantially uncharged lipid. Thus, the lipid mixture may comprise negatively and positively charged lipids.

Compositions of the present invention also include compositions wherein liposomes are formed from a lipid. In some cases, it may be useful to have a composition in which the short oligonucleotide is encapsulated in the liposome. Phospholipids are preferably used for preparing the liposomes according to the present invention and can carry a net positive charge; a net negative charge; or are neutral. The liposomes can be made of one or more phospholipids.

Suitable phospholipids include phosphatidyl cholines and others that are well known to those of skill in the art. Diacetyl phosphate can be employed to confer a negative charge on the liposomes, and stearylamine can be used to confer a positive charge on the liposomes.

Phospholipids from natural sources, such as egg or soybean phosphatidylcholine, brain phosphatidic acid, brain or plant phosphatidylinositol, heart cardiolipin and plant or bacterial phosphatidylethanolamine are preferably not used as the primary phosphatide, *i.e.*, constituting 50% or more of the total phosphatide composition, because of the instability and leakiness of the resulting liposomes.

Thus, one embodiment of the present invention, comprises a liposomal composition of antisense oligonucleotides. The composition includes (a) a liposome which consists essentially of lipids, and (b) a short antisense oligonucleotide, 7 bases to 9 bases in length, that is entrapped in the liposome. In an alternate embodiment, the antisense oligonucleotide may be longer than 9 bases in length and can be 11 bases long and have the sequences represented in SEQ. ID. NO: 6 and SEQ. ID. NO: 7; or can be 15 bases long and have the sequences represented in SEQ. ID. NO: 8, or SEQ. ID. NO: 9.

The antisense oligonucleotide of the invention is preferably composed of a nuclease resistant backbone. Thus, in a preferred embodiment, short antisense p-ethoxy oligonucleotides are contemplated. In alternate embodiments, short antisense phosphorothioate oligonucleotides are contemplated. Furthermore, it is envisioned that any short antisense oligonucleotide that is composed of a nuclease resistant backbone may be used. In yet other embodiments, the use of phosphodiester oligonucleotides are also contemplated.

When the antisense oligonucleotide is a p-ethoxy oligonucleotide, the preferred molar ratio of phospholipid to oligo is between about 5:1 and about 100:1. In a preferred embodiment, for the p-ethoxy oligonucleotides, the molar ratio of phospholipid to oligo is 20:1. A preferred embodiment comprises a) p-ethoxy oligonucleotides and b) the phospholipid dioleoylphosphatidylcholine in a molar ratio of 20:1. When the antisense oligonucleotide is a phosphorothioate oligonucleotide, the preferred molar ratio of phospholipid to oligo is between about 10:1 and about 50:1. When the antisense oligonucleotide is a phosphodiester oligonucleotide, the preferred molar ratio of phospholipid to oligo is less than about 3,000:1.

The short antisense oligonucleotide of the composition may comprises the sequence $^{5'}$GCCATCC$^{3'}$ (SEQ ID NO:2), $^{5'}$TCCTTCC$^{3'}$ (SEQ ID NO:3), $^{5'}$CGCCATCCT^{3} (SEQ ID NO:4), or $^{5'}$ATCCTTCCC$^{3'}$ (SEQ ID NO:5). Alternatively, the antisense oligonucleotide of the

composition may be a 11 base pair sequence which is selected from the group comprising $^{5'}$GCGCCATCCTT$^{3'}$ (SEQ ID NO:6) and $^{5'}$GCCATCCTTCC$^{3'}$ (SEQ ID NO:7). Yet alternatively, the antisense oligonucleotide of the composition may be a 15 base pair sequence which is selected from the group comprising $^{5'}$GTGCGCCATCCTTCC$^{3'}$ (SEQ ID NO:8) and $^{5'}$TGCGCCATCCTTCCC$^{3'}$ (SEQ ID NO:9).

In yet another embodiment, there is provided a composition comprising an expression construct that encodes a short oligonucleotide that is complementary to a Bcl-2 oligonucleotide, wherein the antisense oligonucleotide includes a region complementary to a region of the translation initiation site of Bcl-2 mRNA and wherein the short oligonucleotide is under the control of a promoter that is active in eukaryotic cells. In a specific embodiment, the short oligonucleotide can comprise oligonucleotides of the sequences represented in SEQ. ID. NO: 2, SEQ. ID. NO: 3, SEQ. ID. NO: 4, and SEQ. ID. NO: 5.

An alternative embodiment provides a composition comprising an expression construct that encodes a first oligonucleotide, that is either 11 bases long or 15 bases long, wherein the antisense oligonucleotide includes a region complementary to, or a region that overlaps with a region of the translation initiation site of Bcl-2 mRNA and wherein said first oligonucleotide is under the control of a promoter that is active in cukaryotic cells. In a specific embodiment of the above, the first oligonucleotide can comprise oligonucleotides of the sequences represented in SEQ. ID. NO: 6, SEQ. ID. NO: 7, SEQ. ID. NO: 8, and SEQ. ID. NO: 9.

This invention may be employed to treat a Bcl-2-associated disease. In one embodiment, the invention provides a method for inhibiting a Bcl-2-associated disease comprising: a) obtaining an antisense oligonucleotide having a length of from 7 to 15 bases in length that includes a region complementary to a Bcl-2 oligonucleotide; b) mixing the antisense oligonucleotide with a lipid to form an oligonucleotide-lipid mixture; and c) administering said mixture to a cell. In a specific embodiment, the antisense oligonucleotide is a short oligonucleotide, having a length of from 7 to 9 bases and having the sequence SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5. In other specific embodiments, the antisense oligonucleotide is a 11 base long sequence represented by SEQ. ID. NO: 6 and SEQ. ID. NO: 7; and/or is a 15 base long sequence represented by SEQ. ID. NO: 8 and SEQ. ID. NO: 9. These antisense oligonucleotide or portions thereof may be complementary to a region or a portion of the translation initiation site of Bcl-2 mRNA.

The invention also comprises a method for inhibiting the proliferation of a cancer cell comprising contacting said cancer cell with a composition comprising at least one short

oligonucleotide, 7 bases to 9 bases in length, that is complementary to a portion of a Bcl-2 oligonucleotide. In certain specific embodiments, the short oligonucleotide may be an oligonucleotide having the sequence SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5. In yet another specific embodiment, the invention comprises a method for inhibiting the proliferation of a cancer cell comprising contacting said cancer cell with a composition comprising at least one oligonucleotide, that is 11 bases in length or 15 bases in length, wherein the oligonucleotide has the sequence represented by SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9.

These methods may be applied advantageously to a cancer cell where the cancer cell is a lymphoma cell, a follicular lymphoma cell, a breast cancer cell, a prostate cancer cell, a liver cancer cell, a pancreatic cancer cell, a lung cancer cell, a brain cancer cell, an ovarian cancer cell, a testicular cancer cell, a skin cancer cell, a leukemia cell, a head and neck cancer cell, an esophageal cancer cell, a stomach cancer cell, a kidney cancer cell, a colon cancer cell and a rectal cancer cell.

The composition may further comprise a lipid which is associated with the oligonucleotide, for example, an oligonucleotide encapsulated in a liposome. In a specific embodiment, the contacting takes place in a patient. The patient may be a human. The composition may advantageously be delivered to a human patient in a volume of 0.50-10.0 ml per dose or in an amount of 5-30 mg oligonucleotide per m^2. In a particular regimen, the composition is administered 3 times per week for 8 weeks.

"A" or "an" is defined herein to mean one or more than one.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by

reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Effects of Antisense Oligonucleotide Length on Cell Viability.

FIG. 2. Western Blot Analysis using Bcl-2 Antisense Oligonucleotides. β-actin and Bax are the Negative Controls.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

A. The Present Invention

Bcl-2 is an oncogene with tumorigenic potential due to its capacity to block programmed cell death. The present invention relates to short antisense oligonucleotides directed to portions of the *bcl-2* gene and their use in the treatment of Bcl-2 related diseases. In one embodiment, the present invention employs short antisense oligodeoxynucleotides, that are 9 bases or less, that are associated with a lipid, to inhibit the production of Bcl-2 so that tumor cells can regain the capacity to enter programmed cell death. In another embodiment of the invention, the use of specific antisense oligonucleotides directed to portions of the translational-initiation region of *bcl-2* are disclosed. In this aspect, two 11-mers, bearing SEQ. ID NO: 6, SEQ. ID. NO: 7; and two 15-mers, bearing SEQ ID. NO: 8 and SEQ ID. NO: 9 are shown to inhibit the production of Bcl-2 in CJ cells (which are cells of a transformed follicular lymphoma derived cell-line) and cause the inhibition of cell growth. The present invention may therefore be used to treat hematologic malignancies, both leukemias and lymphomas, including follicular and nonfollicular lymphomas, chronic lymphocytic leukemia, and plasma cell dyscrasias; solid tumors like those associated with breast, prostate, colon, liver, pancreas, lungs, brain, ovary, testis, skin, head and neck, esophageal, stomach, kidney and rectal cancers; and immune disorders, which are associated with Bcl-2 expression.

A specific type of cancer that may be treated by the methods of the present invention is follicular lymphoma. Over 90% of follicular lymphoma patients have a t(14;18) translocation which results in the translocation of the *bcl-2* gene from its normal location in chromosome 18 to the immunoglobulin heavy chain gene locus on chromosome 14. In consequence, the *bcl-2* gene is under the influence of the immunoglobulin heavy chain enhancer, and the Bcl-2 protein is overexpressed. Since *bcl-2* is an oncogene with tumorigenic potential due to its capacity to block programmed cell death, a potential therapy for these follicular lymphomas is to inhibit the production of the Bcl-2 protein. The present invention is unexpected and novel as it uses short

antisense oligonucleotides associated with lipids that are entirely or in-part complementary to portions of the translation initiation site of the Bcl-2 mRNA to inhibit the production of Bcl-2 protein.

It is contemplated that the use of these small antisense molecules, either alone or in conjunction with other antisense molecules, will provide an effective treatment for follicular lymphoma and other cancers. For example, the present invention teaches that treatment with short *bcl-2* antisense oligonucleotides, of from 7 bases to 9 bases, inhibits the growth of CJ cells that are known to overexpress the Bcl-2 protein. In some embodiments, the oligo- or polynucleotides themselves, or expression vectors encoding them, may be employed. The preferred method for delivering these nucleic acids is via liposomes. The invention, in its various embodiments, is described in greater detail, below.

B. Oligonucleotides

The term "antisense" is intended to refer to oligonucleotide or polynucleotide molecules complementary to a portion of a Bcl-2 RNA, or the DNA's corresponding thereto. "Complementary" oligonucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with oligonucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense oligonucleotides, when introduced into a target cell, specifically bind to their target oligonucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

The intracellular concentration of monovalent cation is approximately 160 mM (10 mM Na^+; 150 mM K^+). The intracellular concentration of divalent cation is approximately 20 mM (18 mM Mg^{++}; 2 mM Ca^{++}). The intracellular protein concentration, which would serve to

decrease the volume of hybridization and, therefore, increase the effective concentration of nucleic acid species, is 150 mg/ml. Constructs can be tested *in vitro* under conditions that mimic these *in vivo* conditions.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs for the present invention will include regions complementary to portions of the mRNA start site. One can readily test such constructs simply by testing the constructs *in vitro* to determine whether levels of the target protein are affected. Similarly, detrimental non-specific inhibition of protein synthesis also can be measured by determining target cell viability *in vitro*.

As used herein, the terms "complementary" or "antisense" mean oligonucleotides that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of seven bases in length may be termed complementary when they have a complementary nucleotide for five or six positions out of seven. Naturally, sequences which are "completely complementary" will be sequences which are entirely complementary throughout their entire length and have no base mismatches.

Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, a ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

The oligonucleotides according to the present invention may encode a *bcl-2* gene or a portion of that gene that is sufficient to effect antisense inhibition of protein expression. The oligonucleotides may be derived from genomic DNA, *i.e.*, cloned directly from the genome of a particular organism. In other embodiments, however, the oligonucleotides may be complementary DNA (cDNA). cDNA is DNA prepared using messenger RNA (mRNA) as template. Thus, a cDNA does not contain any interrupted coding sequences and usually contains almost exclusively the coding region(s) for the corresponding protein. In other embodiments, the antisense oligonucleotide may be produced synthetically.

It may be advantageous to combine portions of the genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized

oligonucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

The DNA and protein sequences for *bcl-2* are published in literature by Tsujimoto and Croce (1986) (SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, & SEQ ID NO:14) which is incorporated herein by reference. It is contemplated that natural variants of Bcl-2 exist that have different sequences than those disclosed herein. Thus, the present invention is not limited to use of the provided oligonuclcotide sequence for Bcl-2 but, rather, includes use of any naturally-occurring variants. Depending on the particular sequence of such variants, they may provide additional advantages in terms of target selectivity, *i.e.*, avoid unwanted antisense inhibition of related transcripts. The present invention also encompasses chemically synthesized mutants of these sequences.

As stated above, although the antisense sequences may be full length genomic or cDNA copies, or large fragments thereof, they also may be shorter fragments, or "short oligonucleotides," defined herein as oligonucleotides of from 7 bases to 9 bases. Although shorter oligomers, 7 bases to 9 bases, are easier to make and increase *in vivo* accessibility, numerous other factors are also involved in determining the specificity of base-pairing. For example, both binding affinity and sequencc specificity of an oligonucleotide to its complementary target increase with increasing length. It is contemplated that oligonucleotides of 7, 8, or 9 bases may be used.

In certain embodiments oligonucleotide sequences, longer than 9 bases, for example, of 11 bases and 15 bases bearing SEQ ID NO: 6, SEQ ID. NO: 7, SEQ ID. NO: 8 and SEQ ID. NO: 9 may be used. Other such specific oligonucleotide sequences, longer than 9 bases, for example, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 bases may also be used.

In the present invention any antisense oligonucleotide that is composed of a nuclease resistant backbone and has a favorable binding temperature to allow efficient binding to a target may be used. Thus, a preferred antisense oligonucleotide of this embodiment is a p-ethoxy oligonucleotide. However, phosphodiester oligonucleotides and/or phosphorothioate oligonucleotides are also contemplated. It is also envisioned that any other oligonucleotides with nuclease resistant backbones and favorable binding temperatures may be used.

In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides

which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner *et al.*, 1993).

As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" refers to an RNA-based enzyme capable of targeting and cleaving particular base sequences in both DNA and RNA. Ribozymes can either be targeted directly to cells, in the form of RNA oligonucleotides incorporating ribozyme sequences, or introduced into the cell as an expression vector encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense oligonucleotide. Ribozyme sequences also may be modified in much the same way as described for antisense oligonucleotide. For example, one could incorporate non-Watson-Crick bases, or make mixed RNA/DNA oligonucleotides, or modify the phosphodiester backbone.

Alternatively, the antisense oligo- or polynucleotides of the present invention may be provided as mRNA via transcription from expression constructs that carry nucleic acids encoding the oligonucleotides. Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid encoding an antisense product in which part or all of the nucleic acid sequence is capable of being transcribed. Typical expression vectors include bacterial plasmids or phage, such as any of the pUC or Bluescript™ plasmid series or, as discussed further below, viral vectors adapted for use in eukaryotic cells.

In preferred embodiments, the nucleic acid encodes an antisense oligo- or polynucleotide under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid encoding the antisense oligonucleotides of this invention is not believed to be important, so long as it is capable of expressing the antisense oligonucleotide in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding an antisense oligonucleotide described in this invention adjacent to and under the control of a promoter that is active in the human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of various antisense oligonucleotides described and contemplated in this invention. The use of other viral or mammalian cellular or bacterial phage promoters are well-known to one of skill in the art and the present invention contemplates the use of these promoters as well, provided that the levels of expression of the antisense oligonucleotides are sufficient for the given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of an antisense oligonucleotide can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of an antisense oligonucleotide described herein. For example, a nucleic acid under control of the human PAI-1 promoter results in expression inducible by tumor necrosis factor. Tables 1 and 2 list several elements/promoters which may be employed, in the context of the present invention,

to regulate the expression of antisense constructs. This list is not intended to be exhaustive of all the possible elements involved in the promotion of expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding an antisense oligonucleotide described in this invention in an expression construct (Table 1 and Table 2). Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) also could be used to drive expression of a nucleic acid according to the present invention. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1

PROMOTER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ ß

PROMOTER
ß-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DRα
ß-Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase
Albumin Gene
α-Fetoprotein
τ-Globin
ß-Globin
c-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen

PROMOTER
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

TABLE 2

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
ß-Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester (TPA)
Tumor Necrosis Factor	PHA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone

In certain embodiments of this invention, the delivery of a nucleic acid to a cell may be identified *in vitro* or *in vivo* by including a marker in the expression construct. The marker would result in an identifiable change to the transfected cell permitting easy identification of expression. Enzymes such as herpes simplex virus thymidine kinase (*tk*) (eukaryotic) or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed.

One also may include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Examples include the SV40, globin or adenovirus polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

C. Hybridization

Hybridization is a process by which two complementary nucleic acid strands, such as DNA and DNA, RNA and DNA or RNA and RNA, recognize and bind to each other and form a double stranded structure. Intracellular hybridization is the basis of antisense therapy, which involves the administration/delivery of an antisense nucleic acid to a cell where the antisense molecule finds its complementary target-nucleic acid, which may be either DNA or RNA, and hybridizes to it thereby preventing further transcription or translation of the target-nucleic acid.

The technique of hybridization is also employed to identify nucleic acid products by the nature of the complementarity of a target gene to a hybridization probe. Accordingly, nucleotide sequences may be selected for their ability to selectively form duplex molecules with complementary stretches of genes or RNAs. Depending on the application envisioned, varying conditions of hybridization can be used to achieve varying degrees of selectivity of probe towards target sequence.

For applications requiring high selectivity, one typically will employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating specific genes or detecting specific mRNA transcripts. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

For certain applications, it is appreciated that lower stringency conditions are required. Under these conditions, hybridization may occur even though the sequences of probe/primer and target strand are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to

0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Thus, hybridization conditions can be readily manipulated. and thus will generally be a method of choice depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol. at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 μM $MgCl_2$. at temperatures ranging from approximately 40°C to about 72°C.

The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface to remove non-specifically bound probe/primer molecules, hybridization is detected, or even quantified, by means of the label.

In general, it is envisioned that hybridization of the antisense oligonucleotides of the present invention to the translation initiation site of *bcl-2* mRNA will be the basis of the antisense-gene therapy aimed at Bcl-2 mediated diseases. Intracellular hybridization will prevent the transcription of *bcl-2* mRNA and thereby decrease the Bcl-2 protein content in the cell to which the antisense oligonucleotide is administered to. This will cause the cell to undergo normal apoptosis due to the reduction of cellular Bcl-2 concentration.

D. Lipid Formulations

In a preferred embodiment of the invention, the antisense oligonucleotides and expression vectors may be associated with a lipid. An oligonucleotide associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. The lipid or lipid/oligonucleotide associated compositions of the present invention are not limited to any particular structure in solution. For example, they may be present in a bilayer

structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates which are not uniform in either size or shape.

Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which are well known to those of skill in the art which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes. An example is the lipid dioleoylphosphatidylcholine.

Phospholipids may be used for preparing the liposomes according to the present invention and can carry a net positive charge, a net negative charge or are neutral. Diacetyl phosphate can be employed to confer a negative charge on the liposomes, and stearylamine can be used to confer a positive charge on the liposomes. The liposomes can be made of one or more phospholipids.

In a preferred embodiment, the lipid material is comprised of a neutrally charged lipid. A neutrally charged lipid can comprise a lipid without a charge, a substantially uncharged lipid or a lipid mixture with equal number of positive and negative charges.

In one aspect, the lipid component of the composition comprises a neutral lipid. In another aspect, the lipid material consists essentially of neutral lipids which is further defined as a lipid composition containing at least 70% of lipids without a charge. In other preferred aspects, the lipid material may contain at least 80% to 90% of lipids without a charge. In yet other preferred aspects, the lipid material may comprise about 90%, 95%, 96%, 97%, 98%, 99% or 100% lipids without a charge.

In specific aspects, the neutral lipid comprises a phosphatidylcholine, a phosphatidylglycerol, or a phosphatidylethanolamine. In a preferred aspect, the phosphatidylcholine comprises dioleoylphosphatidylcholine.

In other aspects the lipid component comprises a substantially uncharged lipid. A substantially uncharged lipid is described herein as a lipid composition that is substantially free of anionic and cationic phospholipids and cholesterol. In yet other aspects the lipid component comprises a mixture of lipids to provide a substantially uncharged lipid. Thus, the lipid mixture may comprise negatively and positively charged lipids.

Lipids suitable for use according to the present invention can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma Chemical Co., dicetyl phosphate ("DCP") is obtained from K & K Laboratories

(Plainview, NY); cholesterol ("Chol") is obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, Ala.). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Preferably, chloroform is used as the only solvent since it is more readily evaporated than methanol.

Phospholipids from natural sources, such as egg or soybean phosphatidylcholine, brain phosphatidic acid, brain or plant phosphatidylinositol, heart cardiolipin and plant or bacterial phosphatidylethanolamine are preferably not used as the primary phosphatide, *i.e.*, constituting 50% or more of the total phosphatide composition, because of the instability and leakiness of the resulting liposomes.

"Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes may be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). However, the present invention also encompasses compositions that have different structures in solution than the normal vesicular structure. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

Liposome-mediated oligonucleotide delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the lipid may be associated with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the lipid may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the lipid may be complexed or employed in conjunction with both HVJ and HMG-1. Such expression vectors have been successfully employed in transfer and expression of an oligonucleotide *in vitro* and *in*

vivo and thus are applicable for the present invention. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Liposomes used according to the present invention can be made by different methods. The size of the liposomes varies depending on the method of synthesis. A liposome suspended in an aqueous solution is generally in the shape of a spherical vesicle, having one or more concentric layers of lipid bilayer molecules. Each layer consists of a parallel array of molecules represented by the formula XY, wherein X is a hydrophilic moiety and Y is a hydrophobic moiety. In aqueous suspension, the concentric layers are arranged such that the hydrophilic moieties tend to remain in contact with an aqueous phase and the hydrophobic regions tend to self-associate. For example, when aqueous phases are present both within and without the liposome, the lipid molecules may form a bilayer, known as a lamella, of the arrangement XY-YX. Aggregates of lipids may form when the hydrophilic and hydrophobic parts of more than one lipid molecule become associated with each other. The size and shape of these aggregates will depend upon many different variables, such as the nature of the solvent and the presence of other compounds in the solution.

Liposomes within the scope of the present invention can be prepared in accordance with known laboratory techniques. A novel and preferred method of the invention describes the preparation of liposomes and is described below and in the Examples section. Briefly, p-Ethoxy-oligonucleotides (also referred to as pE oligos) are dissolved in DMSO and the phospholipids (Avanti Polar Lipids, Alabaster, AL), such as for example the preferred neutral phospholipid dioleoylphosphatidylcholine (DOPC), is dissolved in tert-butanol. The lipid is then mixed with the antisense oligonucleotides. In the case of DOPC, the ratio of the lipid to the antisense oligos is 20:1. Tween 20 is added to the lipid:oligo mixture such that Tween 20 is 5% of the combined weight of the lipid and oligo. Excess tert-butanol is added to this mixture such that the volume of tert-butanol is at least 95%. The mixture is vortexed, frozen in a dry ice/acetone bath and lyophilized overnight. The lyophilized preparation is stored at -20°C and can be used upto three months. When required the lyophilized liposomes are reconstituted in 0.9% saline. The average diameter of the particles obtained using Tween 20 for encapsulating the lipid with the oligo is 0.7-1.0 μm in diameter.

Alternatively liposomes can be prepared by mixing liposomal lipids, in a solvent in a container, *e.g.*, a glass, pear-shaped flask. The container should have a volume ten-times greater than the volume of the expected suspension of liposomes. Using a rotary evaporator, the solvent

is removed at approximately 40°C under negative pressure. The solvent normally is removed within about 5 min. to 2 hours, depending on the desired volume of the liposomes. The composition can be dried further in a desiccator under vacuum. The dried lipids generally are discarded after about 1 week because of a tendency to deteriorate with time.

Dried lipids can be hydrated at approximately 25-50 mM phospholipid in sterile, pyrogen-free water by shaking until all the lipid film is resuspended. The aqueous liposomes can be then separated into aliquots, each placed in a vial, lyophilized and sealed under vacuum.

In other alternative methods, liposomes can be prepared in accordance with other known laboratory procedures: the method of Bangham *et al.* (1965), the contents of which are incorporated herein by reference; the method of Gregoriadis, as described in *DRUG CARRIERS IN BIOLOGY AND MEDICINE*, G. Gregoriadis ed. (1979) pp. 287-341, the contents of which are incorporated herein by reference; the method of Deamer and Uster (1983), the contents of which are incorporated by reference; and the reverse-phase evaporation method as described by Szoka and Papahadjopoulos (1978). The aforementioned methods differ in their respective abilities to entrap aqueous material and their respective aqueous space-to-lipid ratios.

The dried lipids or lyophilized liposomes prepared as described above may be dehydrated and reconstituted in a solution of inhibitory peptide and diluted to an appropriate concentration with an suitable solvent, *e.g.*, DPBS. The mixture is then vigorously shaken in a vortex mixer. Unencapsulated nucleic acid is removed by centrifugation at 29,000 × g and the liposomal pellets washed. The washed liposomes are resuspended at an appropriate total phospholipid concentration, *e.g.*, about 50-200 mM. The amount of nucleic acid encapsulated can be determined in accordance with standard methods. After determination of the amount of nucleic acid encapsulated in the liposome preparation, the liposomes may be diluted to appropriate concentrations and stored at 4°C until use.

P-ethoxy oligonucleotides, nucleases resistant analogues of phosphodiesters, are preferred because they are stable in serum. Neutral lipids are also preferred and specifically the lipid dioleoylphosphatidylchoine is preferred. However other lipids such as other phosphatidylcholines, phosphatidylglycerols, and phosphatidylethanolamines may also be useful. In a new and preferred method described herein, the nuclease-resistant oligonucleotides and lipids are dissolved in DMSO and t-butanol respectively. The lipid is then mixed with the oligonucleotides in a ratio of between about 5:1 to about 100:1, and preferably in a ratio of 20:1. The preferred lipid:oligonucleotide ratio for p-ethoxy oligonucleotides and the lipid dioleoylphosphatidylchoine is 20:1. Tween 20 is then added to the mixture to obtain the

liposomes. Excess t-butanol is added and the mixture is vortexed, frozen in a acetone/dry-ice bath, and then lyophilized overnight. The preparation is stored at -20°C and may be used within one month of preparation. When required for use the lyophilized liposomal antisense oligonucleotides are reconstituted in 0.9% saline.

In an alternative embodiment, nuclease-resistant oligonucleotides are mixed with lipids in the presence of excess t-butanol. The mixture is vortexed before being frozen in an acetone/dry ice bath. The frozen mixture is then lyophilized and hydrated with Hepes-buffered saline (1 mM Hepes, 10 mM NaCl, pH 7.5) overnight, and then the liposomes are sonicated in a bath type sonicator for 10 to 15 min. The size of the liposomal-oligonucleotides typically ranges between 200-300 nm in diameter as determined by the submicron particle sizer autodilute model 370 (Nicomp, Santa Barbara, CA).

A pharmaceutical composition comprising the liposomes will usually include a sterile, pharmaceutically acceptable carrier or diluent, such as water or saline solution.

E. Alternative Delivery Systems

Retroviruses The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes - *gag*, *pol*, and *env* - that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene, termed Ψ, functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a Bcl-2 antisense construct as described in this invention is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the *gag*, *pol* and *env* genes but without the LTR and Ψ components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing an inserted DNA, together with the retroviral LTR and Ψ sequences, is introduced into this cell line

(by calcium phosphate precipitation for example), the Ψ sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Adenoviruses Human adenoviruses are double-stranded DNA tumor viruses with genome sizes of approximate 36 kB. As a model system for eukaryotic gene expression, adenoviruses have been widely studied and well characterized, which makes them an attractive system for development of adenovirus as a gene transfer system. This group of viruses is easy to grow and manipulate, and they exhibit a broad host range *in vitro* and *in vivo*. In lytically infected cells, adenoviruses are capable of shutting off host protein synthesis, directing cellular machineries to synthesize large quantities of viral proteins, and producing copious amounts of virus.

The El region of the genome includes E1A and E1B which encode proteins responsible for transcription regulation of the viral genome, as well as a few cellular genes. E2 expression, including E2A and E2B, allows synthesis of viral replicative functions, *e.g.* DNA-binding protein, DNA polymerase, and a terminal protein that primes replication. E3 gene products prevent cytolysis by cytotoxic T cells and tumor necrosis factor and appear to be important for viral propagation. Functions associated with the E4 proteins include DNA replication, late gene expression, and host cell shutoff. The late gene products include most of the virion capsid proteins, and these are expressed only after most of the processing of a single primary transcript from the major late promoter has occurred. The major late promoter (MLP) exhibits high efficiency during the late phase of the infection (Stratford-Perricaudet and Perricaudet, 1991).

A small portion of the viral genome appears to be required in *cis* adenovirus-derived vectors when used in connection with cell lines such as 293 cells. Ad5-transformed human embryonic kidney cell lines (Graham, *et al.*, 1977) have been developed to provide the essential viral proteins *in trans*.

Particular advantages of an adenovirus system for expressing and delivering the antisense oligonucleotides of this invention include (i) the structural stability of recombinant adenoviruses; (ii) the safety of adenoviral administration to humans; and (iii) lack of any known association of adenoviral infection with cancer or malignancies; (iv) the ability to obtain high titers of the recombinant virus; and (v) the high infectivity of adenovirus.

Further advantages of adenovirus vectors over retroviruses include the higher levels of gene expression. Additionally, adenovirus replication is independent of host gene replication, unlike retroviral sequences. Because adenovirus transforming genes in the E1 region can be readily deleted and still provide efficient expression vectors, oncogenic risk from adenovirus vectors is thought to be negligible (Grunhaus & Horwitz, 1992).

In general, adenovirus gene transfer systems are based upon recombinant, engineered adenovirus which is rendered replication-incompetent by deletion of a portion of its genome, such as E1, and yet still retains its competency for infection. Sequences encoding relatively large foreign proteins can be expressed when additional deletions are made in the adenovirus genome. Surprisingly persistent expression of transgenes following adenoviral infection has also been reported.

Other Viral Vectors as Expression Constructs Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpes viruses may be employed. They offer several attractive features for various mammalian cells (Friedman *et al.*, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.* (1991) introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

Non-viral Methods Several non-viral methods for the transfer of expression vectors into cultured mammalian cells also are contemplated in the present invention. These include calcium phosphate precipitation (Graham and van der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990); DEAE-dextran (Gopal, 1985); electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*,

1984); direct microinjection (Harland and Weintraub, 1985); DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979); lipofectamine-DNA complexes; cell sonication (Fecheimer *et al.*, 1987); gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990); polycations; and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

In one embodiment of the invention, the expression construct may simply consist of naked recombinant vector. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. For example, Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding an Bcl-2 antisense oligonucleotide construct may also be transferred in a similar manner *in vivo*.

Another embodiment of the invention for transferring a naked DNA expression vector into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ. DNA encoding a Bcl-2 antisense oligonucleotide as described in this invention may be delivered via this method.

F. Pharmaceutical Compositions and Routes of Administration

Where clinical application of liposomes containing antisense poly- or oligonucleotides is undertaken, it will be necessary to prepare the liposome complex as a pharmaceutical composition appropriate for the intended application. This is also true for expression vectors

encoding the short antisense poly- or oligonucleotides of the invention. Generally. this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate buffers to render the complex stable and allow for uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount of the antisense oligonucleotide encapsulated in a liposome as discussed above, further dispersed in pharmaceutically acceptable carrier or aqueous medium. They may also comprise aqueous compositions of the vectors encoding antisense poly- or oligonucleotides of the invention in one of the vector delivery systems described above. Such compositions also are referred to as inocula. The phrases "pharmaceutically" or "pharmacologically acceptable" refer to compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Solutions of therapeutic compositions can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycol's, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The therapeutic compositions of the present invention are advantageously administered in the form of indictable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like.

Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water,

alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to well known parameters.

Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take thc form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, salve or spray.

The therapeutic compositions of the present invention may include classic pharmaceutical preparations. Administration of therapeutic compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Topical administration would be particularly advantageous for the treatment of skin cancers, to prevent chemotherapy-induced alopecia or other dermal hyperproliferative disorder. Alternatively, administration may be by orthotopic, intradermal subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients. For treatment of conditions of the lungs, the preferred route is aerosol delivery to the lung. Volume of the aerosol is between about 0.01 ml and 0.5 ml. Similarly, a preferred method for treatment of colon-associated disease would be via enema. Volume of the enema is between about 1 ml and 100 ml.

An effective amount of the therapeutic composition is determined based on the intended goal. The term "unit dose" or "dosage" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the protection desired.

Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting the dose include the physical and clinical state of the patient, the route of administration, the intended goal of treatment

(alleviation of symptoms *versus* cure) and the potency, stability and toxicity of the particular therapeutic substance. For the instant application, it is envisioned that the amount of unit dose will range from about 5-30 mg of oligonucleotide.

G. Examples

The following examples are included to demonstrate prcfcrrcd cmbodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modcs for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Synthesis of Oligonucleotides

Antisense oligonucleotides

Nuclease-resistant p-ethoxy oligonucleotides, non-ionic phosphodicstcr analogs, were purchased from Oligo Therapeutics (Willsonville, OR). Alternatively other oligonucleotides such as phosphodiester or phosphorothioate oligonucleotides may also be used and are commercially available. The following oligonucleotide sequences (also listed in Table 3), that corresponds *to bcl-2* antisense, specific for the translation initiation site of human Bcl-2 mRNA, were synthesized and used: $^{5'}$CAGCGTGCGCCATCCTTCCC$^{3'}$ (SEQ ID NO:1), a 20-mer; $^{5'}$GCCATCC$^{3'}$ (SEQ ID NO:2) a 7-mer, $^{5'}$TCCTTCC$^{3'}$ (SEQ ID NO:3), another 7-mer; $^{5'}$CGCCATCCT$^{3'}$ (SEQ ID NO:4), a 9-mer; $^{5'}$ATCCTTCCC$^{3'}$ (SEQ ID NO:5), another 9-mer; $^{5'}$GCGCCATCCTT$^{3'}$ (SEQ ID NO:6), a 11-mer, $^{5'}$GCCATCCTTCC$^{3'}$ (SEQ ID NO:7), another 11-mer; $^{5'}$GTGCGCCATCCTTCC$^{3'}$ (SEQ ID NO:8), a 15-mer; and $^{5'}$TGCGCCATCCTTCCC$^{3'}$ (SEQ ID NO:9), another 15-mer. As a control, a scrambled version of *bcl-2* antisense oligonucleotide, with the sequence: $^{5'}$TCGCCACTCGATCCTGCCCG$^{3'}$ (SEQ ID NO:10) was used.

TABLE 3: Sequences of Various Bcl-2 Antisense Oligonucleotides

SEQ. ID. No. 1	$^{5'}$CAGCGTGCGCCATCCTTCCC$^{3'}$
SEQ ID. NO: 2	$^{5'}$GCCATCC$^{3'}$
SEQ ID. NO: 3	$^{5'}$TCCTTCC$^{3'}$
SEQ ID. NO: 4	$^{5'}$CGCCATCCT$^{3'}$
SEQ ID. NO: 5	$^{5'}$ATCCTTCCC$^{3'}$
SEQ ID. NO: 6	$^{5'}$GCGCCATCCTT$^{3'}$
SEQ ID. NO: 7	$^{5'}$GCCATCCTTCC$^{3'}$
SEQ ID. NO: 8	^{5}GTGCGCCATCCTTCC$^{3'}$
SEQ ID. NO: 9	$^{5'}$TGCGCCATCCTTCCC$^{3'}$
SEQ ID. NO: 10	$^{5'}$TCGCCACTCGATCCTGCCCG$^{3'}$

EXAMPLE 2

Incorporation of Oligonucleotides Into Liposomes

Liposomal p-Ethoxy Oligonucleotides

p-Ethoxy-oligonucleotides (also referred to as pE oligos) are dissolved in DMSO and the phospholipids (Avanti Polar Lipids, Alabaster, AL), for example the preferred neutral phospholipid dioleoylphosphatidylcholine (DOPC), is dissolved in tert-butanol. The lipid is then mixed with the antisense oligonucleotides. In the case of DOPC, the ratio of the lipid to the antisense oligos is 20:1. Tween 20 is added to the lipid:oligo mixture such that Tween 20 is 5% of the combined weight of the lipid and oligo. Excess tert-butanol is added to this mixture such that the volume of tert-butanol is at least 95%. The mixture is vortexed, frozen in a dry ice/acetone bath and lyophilized overnight. The lyophilized preparation is stored at -20°C and can be used upto three months. When required the lyophilized liposomes are reconstituted in

0.9% saline. The average diameter of the particles obtained using Tween 20 for encapsulating the lipid with the oligo is 0.7-1.0 μm in diameter.

EXAMPLE 3

Cell Line and Viability Assays

Cell Line

CJ cells, a human transformed follicular lymphoma cell line bearing the t(14;18) translocation which overexpresses Bcl-2 protein, were used. CJ cells were grown in RPMI 1640 media (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Delivery of liposomal antisense oligonucleotides to cells

Thirty thousand cells/well were seeded in a 96-well plate in 0.1 mL of the respective medium. Cells were incubated with liposomal antisense oligonucleotides at final concentration of 2 to 12 μmol/L at 37° C in a 5% CO_2 incubator. Each experiment was done in triplicate and repeated at least 6-7 times.

Cell viability assay

The viability of the neoplastic cells was measured by the MTS dye (Promega, WI). After 5 days of incubation with liposomal antisense oligonucleotides, 100 μL of fresh medium and 20 μL of MTS dye were added to each well. After incubation for 3-4 hours at 37°C, the plates were read directly on a microplate reader (Molecular Devices, CA) at 490nm. All experiments were analyzed by t-test in which the viabilities of the cells treated with the liposomal antisense oligonucleotides were compared with those of the untreated controls.

Western Blots for Bcl-2 and Bax protein

One hundred thousand cells/well were seeded in a 6-well plate in 3 mL of the respective medium, treated with 8 μmol/L of the 20-mer and the 7-mers (*i.e.* SEQ ID Nos. 1 and 2) of the liposomal antisense oligonucleotides described in Table 3, and incubated at 37°C for three days. Untreated cells were also maintained in culture. Samples were removed on day 3 after the

addition of the liposomal antisense oligonucleotides and lysed in 100 μL of lysis buffer (1% Triton, 150 mmol/L NaCl and 25 mmol/L Tris pH 7.4) at 0°C for 30 minutes. After centrifugation at 12,000 x g for 10 minutes, the supernatants were recovered and normalized for total protein content. The lysates were mixed with sample buffer containing 1% of sodium dodecyl sulfate (SDS) and 1% ß-mercaptoethanol and boiled for 5 minutes. SDS-PAGE was run on 12% polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes and blocked in 5% non-fat dry milk. The membranes were incubated with the anti-human-Bcl-2 monoclonal antibody (Santa Cruz), or rabbit anti-human-Bax polyclonal antibody (Santa Cruz), with mouse anti-actin monoclonal antibody (Sigma). After washing and incubation with a peroxidase-labeled antirabbit or antimouse secondary antibody (Amersham), blots were developed by enhanced chemiluminescence system (Amersham). To estimate the inhibition of Bcl-2 protein and the ratio of Bcl-2/Bax proteins, bands were visualized by enhanced chemiluminescence and densitometric scans were performed on western blots on an AlphaImager 2000 densitometer. The AlphaImage application program was used to determine the ratio of Bcl-2:Actin and Bcl-2:Bax proteins. Results of the Western blot are shown in FIG. 2.

Effect of L-Bcl-2-antisense oligonucleotides ("L-Bcl-2") on lymphoma cell growth

Five days after the addition of 0-12 μmol/L of the L-Bcl-2 antisense oligonucleotides (of SEQ ID Nos. 1-10) to the cells, the viability of tumoral cells was assessed. Cell growth was inhibited in a concentration-dependent manner in CJ cells, which bear the t(14;18) translocation and expresses very high levels of Bcl-2. Sequence-dependent, size-dependent and dose-dependent decreases in cell viabilities were seen in three separate experiments (see data in Table 9).

TABLE 9: Effects of Various Liposomal Bcl-2 Antisense Sequences on the Viability of CJ Cells

Antisense Sequence No. **Concentration of Liposomal Antisense Oligonucleotides**

(Percent Growth and Viability of CJ cells)

	2μM	6μM	10μM
SEQ ID. NO: 1 Regular Bcl-2 AS (20-mer)	75.2	35.4	0
SEQ ID. NO: 10 Scrambled Bcl-2 (Control) (20-mer)	121.0	80.5	85
SEQ ID. NO: 2 (7-mer)	104.1	78.6	57.4
SEQ ID. NO: 3 (7-mer)	62.1	6.0	0
SEQ ID. NO: 4 (9-mer)	87.6	77.3	49.5
SEQ ID. NO: 5 (9-mer)	96.2	66.3	37.6
SEQ ID. NO: 6 (11-mer)	77.4	39.8	8.9
SEQ ID. NO: 7 (11-mer)	63.7	62.3	66.9
SEQ ID. NO: 8 (15-mer)	64.5	54.2	20.0
SEQ ID. NO: 9	76.2	53.0	35.3

(15-mer)			

The ability of the short oligonucleotides to influence cell-viability and growth inhibition were compared with respect to the oligonucleotide encoded by SEQ ID. NO: 1 which is a 20-mer *bcl-2* antisense oligonucleotide. A scrambled *bcl-2* sequence was used as a negative control.

L-bcl-2 selectively downregulates the expression of Bcl-2 protein and cell growth in a dose-dependent manner

The inhibition in cell growth was seen in the CJ follicular lymphoma cell line which bears the t(14;18) translocation. There was no non-specific toxicity in CJ cells exposed to the control oligonucleotide. The growth inhibitory effects could be observed starting at a concentration of 3 μmol/L of L-*bcl-2*, and the inhibitory effects were maximal at 3-8 μmol/L concentration depending on the sequence and length of the specific oligonucleotide (FIG. 1). Furthermore, both the short (7-mer) and the long (20-mer) Bcl-2 could inhibit the expression of Bcl-2 protein to a similar extent (59% *vs* 62%). The Bcl-2-protein inhibition is specific because Bcl-2 did not inhibit Bax and Actin expression. Thus, the inhibition of Bcl-2 protein leads to cell growth inhibition in cells that are dependent on the presence of Bcl-2 protein for maintaining viability.

EXAMPLE 4

In vivo Testing

In an initial round of *in vivo* trials, inventors will use a mice model of human cancer with the histologic features and metastatic potential resembling tumors seen in humans and treat these animals with lipid-associated poly- or oligonucleotide compositions to examine the suppression of tumor development.

These studies are based on the discovery that short *bcl-2* antisense oligonucleotides associated with lipids inhibit the production of the Bcl-2 protein and the growth of t(14;18) translocation bearing cells as described above. The Examples above further show that these lipid formulations inhibit the growth of *bcl-2*-related cancer cells. The current example uses lipid-associated short oligonucleotide formulations, either alone or in combination with

chemotherapeutic drugs, to provide a useful preventive and therapeutic regimen for patients with *bcl-2*-overexpressing cancers.

Mice of a suitable cancer model (*see, e.g.*, McDonnell, 1993) will be treated with doses of the lipid-associated short oligonucleotide compositions or the lipid-associated specific oligonucleotides represented by the sequences in SEQ ID NO: 6, SEQ ID. NO: 7, SEQ ID. NO: 8 and SEQ ID. NO: 9 starting at 8-10 weeks of age or approximately 25 g in weight. The mice used may be transgenic mice bearing the t(14;18) translocation, or they may be nude or SCID mice that were implanted intraperitoneally with human follicular lymphoma cell lines. Several combinations and concentrations of these formulations will be tested. Three groups of mice will be used: untreated mice (*i.e.,* mice injected with buffer only), mice injected with the liposomal short antisense oligos or the lipid-associated specific oligonucleotides represented by the sequences in SEQ ID NO: 6, SEQ ID. NO: 7, SEQ ID. NO: 8 and SEQ ID. NO: 9, and mice injected with liposomal control oligos. The animals will be injected intravenously with liposomal short oligos twice a week. The doses will range between 0-15 mg of liposomal short oligos per kg of mouse in weight. The treatments will be from 6 to 8 weeks.

The effect of the lipid-associated short oligonucleotide compositions or the lipid-associated specific oligonucleotides represented by the sequences in SEQ ID NO: 6, SEQ ID. NO: 7, SEQ ID. NO: 8 and SEQ ID. NO: 9 on the development of follicular lymphoma tumors will be compared with the control group by measuring tumor size, mouse survival, B cell hyperplasia, and Bcl-2 expression. It is predicted that, unlike the control groups of mice that will develop tumors, the testing group of mice will have decreased Bcl-2 expression, B cell hyperplasia, and tumor size, as well as prolonged survival. The group treated with liposomal control oligos should have no such effects.

EXAMPLE 5

Clinical Trials

This example is concerned with the development of human treatment protocols using the lipid-associated short oligonucleotide compositions or the lipid-associated specific oligonucleotides represented by the sequences in SEQ ID NO: 6, SEQ ID. NO: 7, SEQ ID. NO: 8 and SEQ ID. NO: 9. These lipid formulations will be of use in the clinical treatment of various *bcl-2*-overexpressing cancers and diseases in which transformed or cancerous cells play a role. Such treatment will be particularly useful tools in anti-tumor therapy, for example, in treating

patients with follicular lymphoma. This treatment will also be useful in treating other conditions that are mediated by *bcl-2* over-expression and resistant to conventional regimens and treatments such as hematologic malignancies, both leukemias and lymphomas, including follicular and nonfollicular lymphomas, chronic lymphocytic leukemia, and plasma cell dyscrasias; solid tumors like those associated with breast, prostate and colon cancer; and immune disorders.

The various elements of conducting a clinical trial, including patient treatment and monitoring, will be known to those of skill in the art in light of the present disclosure. The following information is being presented as a general guideline for use in establishing lipid-associated short oligonucleotide compositions alone or in combinations with other anti-cancer drugs in clinical trials.

Candidates for the phase 1 clinical trial will be patients on which all conventional therapies have failed. Liposomal Bcl-2 antisense short oligos and/or the lipid-associated specific oligonucleotides represented by the sequences in SEQ ID NO: 6, SEQ ID. NO: 7, SEQ ID. NO: 8 and SEQ ID. NO: 9 will be administered to them intravenously on a tentative weekly basis. To monitor disease course and evaluate the anti-tumor responses, it is contemplated that the patients should be examined for appropriate tumor markers every month. To assess the effectiveness of the drug, the following parameters will be monitored: tumor size and bone marrow infiltration of the cancer cells. Tests that will be used to monitor the progress of the patients and the effectiveness of the treatments include: physical exam, X-ray, blood work and other clinical laboratory methodologies. In addition, peripheral blood and bone marrow samples will be drawn to assess the modification of the target protein expression. The doses given in the phase 1 study will be escalated as is done in standard phase 1 clinical phase trials, i.e. doses will be escalated until maximal tolerable ranges are reached.

Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by complete disappearance of evidence of cancer cells for at least 2 months. Whereas a partial response may be defined by a 50% reduction of cancer cells for at least 2 months.

EXAMPLE 6

Human Treatment and Clinical Protocols

This example describes a protocol to facilitate the treatment of *bcl-2*-mediated diseases using lipid-associated short oligonucleotide compositions and/or the lipid-associated specific

oligonucleotides represented by the sequences in SEQ ID NO: 6, SEQ ID. NO: 7, SEQ ID. NO: 8 and SEQ ID. NO: 9 alone or in combination with other anti-cancer drugs.

Typically, patients that are candidates for treatment are those with follicular lymphoma although patients with hematologic malignancies, both leukemias and lymphomas; solid tumors like those associated with breast, prostate and colon cancer; and immune disorders may also be treated with the methods of this invention. The typical course of treatment will vary depending upon the individual patient and disease being treated in ways known to those of skill in the art. For example, a patient with follicular lymphoma might be treated in eight week cycles, although longer duration may be used if no adverse effects are observed with the patient, and shorter terms of treatment may result if the patient does not tolerate the treatment as hoped. Each cycle will consist of between 20 and 35 individual doses spaced equally, although this too may be varied depending on the clinical situation.

A patient presenting a *bcl-2*-mediated condition, like follicular lymphoma, may be treated using the following protocol. Patients may, but need not, have received previous chemo-, radio- or gene therapeutic treatments. Optimally the patient will exhibit adequate bone marrow function (defined as peripheral absolute granulocyte count of > 2,000/mm^3 and platelet count of 100, 000/mm^3, adequate liver function (bilirubin 1.5mg/dl) and adequate renal function (creatinine 1.5mg/dl).

The over-expression of *bcl-2* is typically monitored before, during, and after the therapy. A composition of the present invention is typically administered orally or parenterally in dosage unit formulations containing standard, well known non-toxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intra-arterial injection, or infusion techniques. The lipid-associated short oligo-nucleotide compositions and/or the lipid-associated specific oligonucleotides represented by the sequences in SEQ ID NO: 6, SEQ ID. NO: 7, SEQ ID. NO: 8 and SEQ ID. NO: 9 may be delivered to the patient before, after or concurrently with the other anti-cancer agents.

A typical treatment course may comprise about six doses delivered over a 7 to 21 day period. Upon election by the clinician the regimen may be continued with six doses every three weeks or on a less frequent (monthly, bimonthly, quarterly etc.) basis. Of course, these are only exemplary times for treatment, and the skilled practitioner will readily recognize that many other time-courses are possible.

To kill *bcl-2*-overexpressing cancer cells using the methods and compositions described in the present invention one will generally contact a target cell with the lipid-associated formulations described previously. These compositions will be provided in an amount effective to kill or inhibit the proliferation of the cell.

Regional delivery of the lipid-associated formulations will be an efficient method for delivering a therapeutically effective dose to counteract the clinical disease. Alternatively systemic delivery may be appropriate. The therapeutic composition of the present invention may be administered to the patient directly at the site of the tumor. This is in essence a topical treatment of the surface of the cancer. The volume of the composition should usually be sufficient to ensure that the entire surface of the tumor is contacted by the lipid-associated short oligonucleotide composition and/or the lipid-associated specific oligonucleotides represented by the sequences in SEQ ID NO: 6, SEQ ID. NO: 7, SEQ ID. NO: 8 and SEQ ID. NO: 9.

In one embodiment, administration simply entails injection of the therapeutic composition into the tumor. In another embodiment, a catheter is inserted into the site of the tumor and the cavity may be continuously perfused for a desired period of time.

Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by complete disappearance of evidence of cancer cells for at least 2 months. Whereas a partial response may be defined by a 50% reduction of cancer cells for at least 2 months.

Of course, the above-described treatment regimes may be altered in accordance with the knowledge gained from clinical trials such as those described in Example 5. Those of skill in the art will be able to take the information disclosed in this specification and optimize treatment regimes based on the clinical trials described in the specification.

* * * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be

achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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CLAIMS

1. A composition comprising an antisense oligonucleotide having a length of from 7 to 9 bases and complementary to a Bcl-2 oligonucleotide, and a lipid component.

2. The composition of claim 1, wherein the antisense oligonucleotide includes a region complementary to a region of the translation initiation site of Bcl-2 mRNA.

3. The composition of claim 1, wherein the lipid component comprises liposomes.

4. The composition of claim 3, wherein the antisense oligonucleotide is encapsulated in liposomes.

5. The composition of claim 1, wherein the lipid component comprises a neutral lipid.

6. The composition of claim 5, wherein the neutral lipid comprises a phosphatidylcholine, a phosphatidylglycerol, or a phosphatidylethanolamine.

7. The composition of claim 6, wherein the phosphatidylcholine comprises dioleoylphosphatidylcholine.

8. The composition of claim 1, wherein the lipid component comprises a mixture of lipids to provide a substantially uncharged lipid.

9. The composition of claim 8, wherein the lipid mixture comprises negatively and positively charged lipids.

10. The composition of claim 1, wherein the antisense oligonucleotide is a phosphodiester oligonucleotide.

11. The composition of claim 1, wherein the antisense oligonucleotide is a nuclease-resistant oligonucleotide.

12. The composition of claim 11, wherein said nuclease-resistant oligonucleotide is a p-ethoxy oligonuclcotide.

13. The composition of claim 11, wherein said nuclease-resistant oligonucleotide is a phosphorothioate oligonucleotide.

14. The composition of claim 1, wherein the antisense oligonucleotide comprises the sequence $^{5'}$GCCATCC$^{3'}$ (SEQ ID NO:2).

15. The composition of claim 1, wherein the antisense oligonucleotide comprises the sequence $^{5'}$TCCTTCC$^{3'}$ (SEQ ID NO:3).

16. The composition of claim 1, wherein the antisense oligonucleotide comprises the sequence $^{5'}$CGCCATCCT^{3} (SEQ ID NO:4).

17. The composition of claim 1, wherein the antisense oligonucleotide comprises the sequence $^{5'}$ATCCTTCCC$^{3'}$ (SEQ ID NO:5).

18. A composition comprising an antisense oligonucleotide and a lipid component, wherein the antisense oligonucleotide has a 11 base pair sequence and is selected from the group comprising $^{5'}$GCGCCATCCTT$^{3'}$ (SEQ ID NO:6) and ^{5} GCCATCCTTCC$^{3'}$ (SEQ ID NO:7).

19. A composition comprising an antisense oligonucleotide and a lipid component wherein the antisense oligonuclcotide has a 15 base pair sequence and is selected from the group comprising $^{5'}$GTGCGCCATCCTTCC$^{3'}$ (SEQ ID NO:8) and $^{5'}$TGCGCCATCCTTCCC$^{3'}$ (SEQ ID NO:9).

20. A method of inhibiting a Bcl-2-associated disease comprising:

a) obtaining an antisense oligonucleotide having a length of from 7 to 15 bases that is complementary to a Bcl-2 oligonucleotide;

b) mixing the antisense oligonucleotide with a lipid to form an oligonucleotide-lipid mixture; and

c) administering said mixture to a cell.

21. The method of claim 20, wherein the cell is a cancer cell.

22. The method of claim 21, wherein said cancer cell is a follicular lymphoma cell, a breast cancer cell, a prostate cancer cell, liver cancer cell, a pancreatic cancer cell, a lung cancer cell, a brain cancer cell, an ovarian cancer cell, a testicular cancer cell, a skin cancer cell, a leukemia cell, a head and neck cancer cell, an esophageal cancer cell, a stomach cancer cell, a kidney cancer cell, a colon cancer cell and a rectal cancer cell.

23. The method of claim 20, wherein the lipid component comprises a liposome.

24. The method of claim 23, wherein the liposome encapsulates the antisense oligonucleotide.

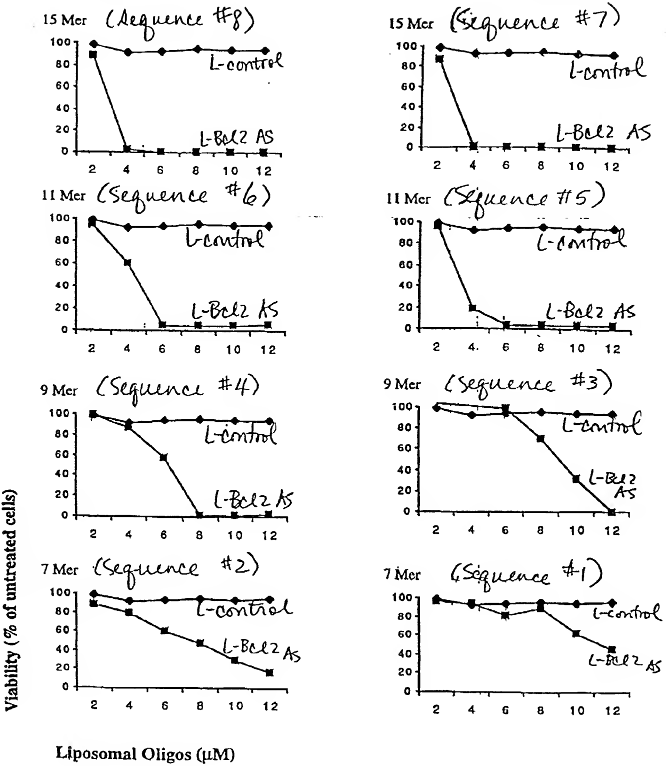
25. The mcthod of claim 20, wherein said cell is in an animal.

26. The method of claim 25, wherein said animal is a human.

27. The method of claim 26, wherein said association is delivered to said human in a volume of 0.50-10.0 ml per dose.

28. The method of claim 26, wherein said association is delivered to said human in an amount of from about 5 to about 30 mg oligonucleotide per m^2.

29. The method of claim 26, wherein said association is administered three times per week for eight weeks.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11 Mer (Sequence #6)
11 Mer (Sequence #5)
9 Mer (Sequence #4)
9 Mer (Sequence #3)
7 Mer (Sequence #2)
7 Mer (Sequence #1)
L-control
L-Bcl2 AS
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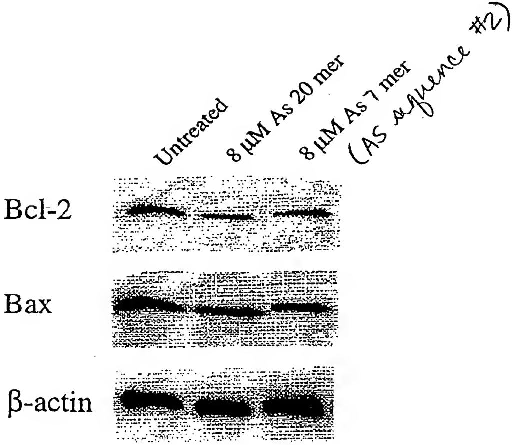
20
40
60
80
100
2
4
6
8
10
12
Viability (% of untreated cells)
Liposomal Oligos (μM)

Fig. 1.



Bcl-2 inhibition (% of untreated cells) — 59 66

FIG. 2.

SEQUENCE LISTING

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LOPEZ-BERESTEIN, GABRIEL
GUTIERREZ-PUENTE, YOLANDA

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ccgtagtcgc gccgccgcgc aggaccagga ggaggagaaa gggtgcgcag cccggaggcg 240

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Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp
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Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala
 65                  70                  75                  80

Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala
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Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe
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Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
        115                 120                 125

Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp
    130                 135                 140

Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu
145                 150                 155                 160

Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
                165                 170                 175

Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
            180                 185                 190

Gly Gly Trp Val Gly Ala Ser Gly Asp Val Ser Leu Gly
        195                 200                 205
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